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(71) Applicant: SCHERING CORPORATION [US/US]; 2000 Galloping Hill Road, Kenilworth, NJ 07033-0530 (US).

(72) Inventors: BAKKER, Alexander, B., H.; 344 Duncan Street, San Francisco, CA 94131 (US). PHILLIPS, Joseph, H., Jr.; 1511 Walnut Drive, Palo Alto, CA 94303 (US). LANIER, Lewis, L.; 1528 Frontero Avenue, Los Altos, CA 94024 (US).

(74) Agents: THAMPOE, Immac, J. et al.; Schering-Plough Corporation, Patent Dept., K-6-1 1990, 2000 Galloping Hill Road, Kenilworth, NJ 07033-0530 (US).

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(57) Abstract

The purification and isolation of various genes which encode mammalian cell surface polypeptides. Nucleic acids, proteins, antibodies, and other reagents useful in modulating development of cells, e.g., lymphoid and myeloid, are provided, along with methods for their use.

VIII. Uses

The present invention provides reagents which will find use in diagnostic applications as described elsewhere herein, e.g., in the general description for developmental or physiological abnormalities, or below in the description of kits for diagnosis.

Many of the receptors important in the activation of leukocytes (including the T cell antigen receptor, and immunoglobulin and Fc receptors) lack intrinsic signaling properties, but transmit their signals by coupling non-covalently with other membrane proteins that contain immunoreceptor tyrosine-based activation motifs (ITAM, YxxL- 6 to 8 amino acid spacer -YxxL) in their cytoplasmic domains. For example, the T cell antigen receptor is associated with the CD3 gamma, delta, epsilon, and zeta proteins that contain ITAM sequences. Similarly, surface immunoglobulin on B cells is associated with CD79A and CD79B that contain ITAM and are required for signal transduction. The Fc receptors for IgG (CD16) on NK cells associates with CD3 zeta or the IgE Fc receptor-gamma subunit (both containing ITAM) and the high affinity IgE receptor on mast cells associated with the IgE Fc receptor-gamma subunit. Therefore, associated proteins containing ITAM represent a general strategy in the assembly of activating receptors on leukocytes.

Recently, several new families of leukocyte receptors have been identified that are structurally diverse. Certain isoforms of the KIR, ILT/MIR, Ly49, and CD94/NKG2 family of receptors have been implicated in positive signaling; however, these molecules (e.g. KIR-NKAT5, KIR-c139, ILT1, gp91/PIR, and CD94) lack sequences in their cytoplasmic domains that would be consistent with positive signaling capability.

Given that T cell antigen receptors, immunoglobulin receptors, and Fc receptors all achieve signaling function by association with another small subunit containing ITAM, it is likely that these other leukocyte receptors might use a similar strategy.

Therefore, available sequence databases were searched with protein sequences of human and mouse CD3 gamma, delta, epsilon, and zeta, and IgE Fc receptor-gamma chain. An EST designated LVA03A was identified that encodes a putative membrane protein of ~12 kd with an acidic residue (D) in the transmembrane segment and a perfect ITAM sequence in the cytoplasmic domain. Cysteine residues in the short extracellular domain suggest the molecule might be expressed as a disulfide-bonded dimer. Distribution studies indicate the gene is transcribed in macrophages, dendritic cells, some T cells, and NK cells. This protein has been designated DNAX Activating Protein 12 (DAP12). An analogous gene was also identified, designated DAP10, which possesses ITIM motifs.

Receptors containing ITAM have all been important in inducing leukocyte function (e.g., T cell antigen receptor, immunoglobulin receptor, Fc receptor). Therefore, it is probably that DAP12 will have an important role in signal transduction in leukocytes. Agonists and antagonists of DAP12 should provide useful in either potentiating or inhibiting immune responses (i.e., proliferation, cytokine production, inducing apoptosis, or triggering cell-mediated cytotoxicity), respectively.

Receptors containing the YxxM motif have been identified as important in certain signaling molecules, e.g., CD28, CTLA-4, and CD19. Therefore, it is probably that DAP10 will have an important role in signal transduction. Agonists and antagonists of DAP10 should provide useful in either potentiating or inhibiting

immune responses (i.e., proliferation, cytokine production, inducing apoptosis, or triggering cell-mediated cytotoxicity), respectively.

It is anticipated that DAP12 may non-covalently associate with several different membrane receptors, for example, but not necessarily limited to T cell antigen receptor, the pre-T cell antigen receptor, the immunoglobulin receptor, Fc receptors, the KIR family of receptors, the ILT/MIR family of receptors, the LAIR family of receptors, the gp91/PIR family of receptors, the Ly49 family of receptors (specifically Ly49D and Ly49H), and the CD94/NKG2 family of receptors. Among these is the MDL-1. Therefore, reagents to affect DAP12 interaction with said receptors may either enhance or suppress the function of these molecules for therapeutic intervention (i.e., augment immunity for vaccination or immunodeficiency diseases or suppress immune responses in the case of autoimmune diseases or transplantation). Combinations of DAP with any one of these receptors will be useful, e.g., for drug screening for interrupters of the interaction and subsequent signaling, as will antibodies to the structural complexes arising form their interaction.

The DAP12 may be playing a role in Beta2 like integrin signaling. It is clear that Beta2 integrin can transmit a P Tyr kinase dependent signal involving Syk. In Syk knockouts, Beta2 does not signal. The pathway also probably involves FcγR (in Monocytes/Macrophages and B cells) as a negative regulator. However, there is no known way for Syk to associate with Beta2 integrins as they have no ITAM containing sequences in there cytoplasmic domains. Moreover, there is no evidence that the known ITAM containing proteins can associate with Beta2. Thus, DAP12 would be a prime candidate or prototype for one that would associate with Beta2.

This invention also provides reagents with significant therapeutic value. The human DAP12 or DAP10 (naturally occurring or recombinant), fragments thereof and antibodies thereto, along with compounds identified as having binding affinity to primate DAP, should be useful in the treatment of conditions associated with abnormal B cell response, including abnormal proliferation, e.g., cancerous conditions, or degenerative conditions. Abnormal proliferation, regeneration, degeneration, and atrophy may be modulated by appropriate therapeutic treatment using the compositions provided herein. For example, a disease or disorder associated with abnormal expression or abnormal triggering of DAP12 should be a likely target for an agonist or antagonist of the antigen. DAP12 likely plays a role in activation or regulation of immune cells, which affect immunological responses, e.g., autoimmune disorders or allergic responses.

In addition, the DAP:DAP binding partner interaction may be involved in T, NK, DC, or monocyte cell interactions that permit the activation, proliferation, and/or differentiation interacting cells. If so, treatment may result from interference with the DAP:DAP binding partner signal transduction, particularly potentiating or inhibiting immune responses such as proliferation, cytokine production, inducing apoptosis, or triggering cell-mediated cytotoxicity. Blocking of the signal may be effected, e.g., by soluble DAP or antibodies to DAP, or drugs which disrupt the functional interaction of the DAP with its receptor complex partner.

Other abnormal developmental conditions are known in each of the cell types shown to possess DAP12 or DAP10 mRNA by Northern blot analysis, e.g., lymphocytes, NK, monocytes, and dendritic cells. See Berkow (ed.) The Merck Manual of Diagnosis and Therapy, Merck & Co., Rahway, N.J.; and Thorn, et al. Harrison's Principles of

Internal Medicine, McGraw-Hill, N.Y. For example, therapeutic immunosuppression may be achieved by blocking T lymphocyte and B lymphocyte interaction through this molecule. It will represent an important therapy for controlling autoimmune diseases and graft rejection during transplantation. The blockage may be effected with blocking binding compositions, e.g., neutralizing antibodies.

Recombinant DAP or DAP antibodies can be purified and then administered to a patient. These reagents can be combined for therapeutic use with additional active ingredients, e.g., in conventional pharmaceutically acceptable carriers or diluents, e.g., immunogenic adjuvants, along with physiologically innocuous stabilizers and excipients. These combinations, and compositions provided, can be sterile filtered and placed into dosage forms as by lyophilization in dosage vials or storage in stabilized aqueous preparations. This invention also contemplates use of antibodies or binding fragments thereof which are not complement binding.

Drug screening using DAP or fragments thereof can be performed to identify compounds having binding affinity to a DAP, including isolation of associated components. Subsequent biological assays can then be utilized to determine whether the compound has intrinsic stimulating activity and is therefore a blocker or antagonist in that it blocks signaling. Likewise, a compound having intrinsic stimulating activity can activate the antigen and is thus an agonist in that it simulates the activity of a DAP. This invention further contemplates the therapeutic use of antibodies to DAP as antagonists. This approach should be particularly useful with other DAP or MDL species variants.

The quantities of reagents necessary for effective therapy will depend upon many different factors, including means of administration, target site,

physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used in vitro may provide useful guidance in the amounts useful for in situ administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, e.g., in Gilman, et al. (eds. 1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Penn. Methods for administration are discussed therein and below, e.g., for oral, intravenous, intraperitoneal, or intramuscular administration, transdermal diffusion, and others. Pharmaceutically acceptable carriers will include water, saline, buffers, and other compounds described, e.g., in the Merck Index, Merck & Co., Rahway, New Jersey. Dosage ranges would ordinarily be expected to be in amounts lower than 1 mM concentrations, typically less than about 10 μM concentrations, usually less than about 100 nM, preferably less than about 10 pM (picomolar), and most preferably less than about 1 fM (femtomolar), with an appropriate carrier. Slow release formulations, or a slow release apparatus will often be utilized for continuous administration.

Human DAP or MDL, fragments thereof, and antibodies to it or its fragments, antagonists, and agonists, may be administered directly to the host to be treated or, depending on the size of the compounds, it may be desirable to conjugate them to carrier proteins such as ovalbumin or serum albumin prior to their administration. Therapeutic formulations may be administered in many conventional dosage formulations. While it is possible for the active ingredient to be administered alone, it is preferable to present it as a pharmaceutical formulation.

Formulations typically comprise at least one active ingredient, as defined above, together with one or more acceptable carriers thereof. Each carrier should be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Formulations include those suitable for topical, oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in unit dosage form, in sterile forms, or may be prepared by many methods well known in the art of pharmacy. See, e.g., Gilman, et al. (eds. 1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Penn. The therapy of this invention may be combined with or used in association with other agents.

Both the naturally occurring and the recombinant forms of the DAP or MDL antigens of this invention are particularly useful in kits and assay methods which are capable of screening compounds for binding activity to the proteins. Several methods of automating assays have been developed in recent years so as to permit screening of tens of thousands of compounds in a short period. See, e.g., Fodor, et al. (1991) Science 251:767-773, which describes means for testing of binding affinity by a plurality of defined polymers synthesized on a solid substrate. The development of suitable assays can be greatly facilitated by the availability of large amounts of purified, soluble DAP or MDL as provided by this invention.

For example, antagonists can normally be found once a DAP or MDL has been structurally defined. Testing of potential antagonists is now possible upon the development of highly automated assay methods using a purified DAP or MDL. In particular, new agonists and

antagonists will be discovered by using screening techniques made available herein. Of particular importance are compounds found to have a combined binding affinity for multiple DAP12, DAP10, or MDL-1 proteins, e.g., compounds which can serve as antagonists for allelic variants of DAP or MDL.

Moreover, since the signaling through the DAP:DAP binding partner may function in combination with other signals, combination therapy with such pathways will also be considered. Thus, antagonism of multiple signal pathways, or stimulation with multiple pathways may be useful. Moreover, with the association of the DAP12 with MDL-1, and possibly also with DAP10, various combinations of the described genes may be important.

This invention is particularly useful for screening compounds by using the recombinant antigens in any of a variety of drug screening techniques. The advantages of using a recombinant protein in screening for specific compounds include: (a) improved renewable source of the DAP12 from a specific source; (b) potentially greater number of antigen molecules per cell giving better signal to noise ratio in assays; and (c) species variant specificity (theoretically giving greater biological and disease specificity).

One method of drug screening utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant DNA molecules expressing the DAP and/or MDL. Cells may be isolated which express a DAP in isolation from others, or in combination with its receptor complex partner. Such cells, either in viable or fixed form, can be used for standard antigen/partner binding assays. See also, Parce, et al. (1989) Science 246:243-247; and Owicki, et al. (1990) Proc. Nat'l Acad. Sci. USA 87:4007-4011, which describe sensitive methods to detect cellular responses. Competitive assays are particularly useful, where the cells (source of DAP) are contacted and

incubated with a labeled compound having known binding affinity to the antigen, and a test compound whose binding affinity to the DAP is being measured. The bound compound and free compound are then separated to assess the degree of binding. The amount of test compound bound is inversely proportional to the amount of labeled compound binding measured. Many techniques can be used to separate bound from free compound to assess the degree of binding. This separation step could typically involve a procedure such as adhesion to filters followed by washing, adhesion to plastic followed by washing, or centrifugation of the cell membranes. Viable cells could also be used to screen for the effects of drugs on DAP mediated functions, e.g., second messenger levels, i.e., Ca^{++}; cell proliferation; inositol phosphate pool changes; and others. Some detection methods allow for elimination of a separation step, e.g., a proximity sensitive detection system. Calcium sensitive dyes will be useful for detecting Ca^{++} levels, with a fluorimeter or a fluorescence cell sorting apparatus.

Another method utilizes membranes from transformed eukaryotic or prokaryotic host cells as the source of the human DAP or MDL. These cells are stably transformed with DNA vectors directing the expression of human DAP or MDL antigen. Essentially, the membranes would be prepared from the cells and used in a receptor complex binding assay such as the competitive assay set forth above.

Still another approach is to use solubilized, unpurified or solubilized, purified DAP from transformed eukaryotic or prokaryotic host cells. This allows for a "molecular" binding assay with the advantages of increased specificity, the ability to automate, and high drug test throughput.

Another technique for drug screening involves an approach which provides high throughput screening for

compounds having suitable binding affinity to human DAP or MDL and is described in detail in Geysen, European Patent Application 84/03564, published on September 13, 1984. First, large numbers of different small peptide test compounds are synthesized on a solid substrate, e.g., plastic pins or some other appropriate surface, see Fodor, et al. (1991). Then all the pins are reacted with solubilized, unpurified or solubilized, purified DAP, and washed. The next step involves detecting bound DAP.

Rational drug design may also be based upon structural studies of the molecular shapes of the DAP or MDL and other effectors. Effectors may be other proteins which mediate other functions in response to receptor complex binding, or other proteins which normally interact with the antigen. One means for determining which sites interact with specific other proteins is a physical structure determination, e.g., x-ray crystallography or 2 dimensional NMR techniques. These will provide guidance as to which amino acid residues form molecular contact regions. For a detailed description of protein structural determination, see, e.g., Blundell and Johnson (1976) Protein Crystallography, Academic Press, New York.

Purified DAP or MDL can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-neutralizing antibodies to these antigens can be used as capture antibodies to immobilize the respective DAP or MDL on the solid phase.

and metaphase photographed. R-banding is then performed by the fluorochrome-photolysis-Giemsa (FPG) method and metaphases re-photographed before analysis.

The genomic organization of human DAP12 consists of 5 exons spanning ~4 kb on chromosome 19q13.1. The human KIR genes (Baker, et al. (1995) Chromosome Research 3:511) and the related LAIR (Meyaard, et al. (1997) Immunity 7:283-290, and ILT/MIR (Wagtmann, et al. (1997) Current Biology 7:615-618) genes are all located nearby on chromosome 19q13.4.

XI. DAP and MDL biology

DAP12 is a disulfide-bonded homodimer, containing an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain, that is predominantly expressed in NK cells, monocytes, and dendritic cells. This molecule non-covalently associates with membrane glycoproteins of the killer cell inhibitory receptor (KIR) family that lack immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domain. Cross-linking KIR2DS2-DAP12 complexes expressed in transfectants results in cellular activation, as demonstrated by tyrosine-phosphorylation of cellular proteins and up-regulation of early activation antigens. Phosphorylated DAP12 peptides bind ZAP-70 and Syk protein tyrosine kinases, suggesting an activation pathway similar to the T and B cell antigen receptors.

NK cells express membrane receptors of the immunoglobulin and C-type lectin superfamilies that recognize MHC class I and inhibit NK cell-mediated cytotoxicity. Lanier (1997) Immunity 6:371-378. These inhibitory receptors (including human KIR, human CD94/NKG2A, and rodent Ly49) possess ITIM in their cytoplasmic domains that recruit SH2-domain containing protein tyrosine phosphatases (SHP) 1 or 2, resulting in

inactivation of NK cell function. Burshtyn, et al. (1996) Immunity 4:77-85; Olcese, et al. (1996) J. Immunol. 156:4531-4534; and Houchins, et al. (1997) J. Immunol. 158:3603-3609. Certain isoforms of the KIR, Ly49, and CD94/NKG2 receptors lack ITIM sequences and it has been proposed that these 'non-inhibitory' receptors may activate, rather than inhibit, NK cell function. Houchins, et al. (1997) J. Immunol. 158:3603-3609; Biassoni, et al. (1996) J. Exp. Med. 183:645-650; and Mason, et al. (1996) J. Exp. Med. 184:2119-2128. When the non-inhibitory KIR2DS2 molecule was expressed by transfection in the RBL-2H3 basophilic leukemia no cellular activation was observed when the receptors were ligated, suggesting that these 'non-inhibitory' NK receptors may lack intrinsic signaling properties. Bléry, et al. (1997) J. Biol. Chem. 272:8989-8996.

Recently, Olcese, et al. (1997) J. Immunol. 158:5083-5086, reported that an unknown phosphoprotein of ~12 kD, expressed as a disulfide-bonded dimer, was co-immunoprecipitated with a non-inhibitory KIR2DS2 glycoprotein from NK cell lysates. Cell surface Ig receptors, T cell antigen receptors (TcR), and certain Fc receptors (FcR) non-covalently associate with small transmembrane proteins (e.g. CD3δ, γ, ε, ζ subunits, CD79α, β, FcεRI-γ) containing ITAM sequences (D/ExxYxxL/I - x_{6-8}- YxxL/I; Reth (1989) Nature 338:383-384) that are required for signal transduction by these receptor complexes. Chan, et al. (1994) Ann. Rev. Immunol. 12:555-592. Therefore, it seems likely that these non-inhibitory NK cell receptors might require an associated protein with similar properties to mediate positive signal transduction.

A database of expressed tag sequences (EST) from a large panel of cDNA libraries was searched with a TBLASTN algorithm program for molecules bearing homology with the human CD3δ, γ, ε, ζ and FcεRI-γ protein sequences. An EST

from a human CD1+ dendritic cell library was selected for further study based on identification of an ITAM in this molecule. Sequencing of the 604 bp cDNA revealed an open reading frame of 339 nucleotides, encoding a putative type I membrane protein of 113 amino acids (see SEQ ID NO: 1 and 2). The protein, designated DAP12, is composed of a 27 aa leader, 14 aa extracellular domain, 24 aa transmembrane segment, and 48 aa cytoplasmic region. Although DAP12 has less than 25% homology with the human CD3δ, γ, ε, ζ and FcεRI-γ proteins, the cytoplasmic domain contains the peptide, ESPYQELQGQRSDVYSDL (see SEQ ID NO: 2), that precisely corresponds to the prototype ITAM consensus sequence. Potential sites for phosphorylation by protein kinase C (residues 79-81 and 107-109) and casein kinase II (residues 85-88) are also present in the DAP12 cytoplasmic region. The transmembrane region contains a charged amino acid (D), also conserved in the transmembrane domain of the CD3 subunits. A potential murine homolog of DAP12 is ~70% homologous with the human DAP12 protein and has a conserved D residue in the transmembrane region, conserved C residues in the extracellular domain and an ITAM in the cytoplasmic region.

A conspicuous feature of the non-inhibitory KIR (Biassoni, et al. (1996) J. Exp. Med. 183:645-650), Ly49D and Ly49H (Mason, et al. (1996) J. Exp. Med. 184:2119-2128), CD94 (Chang, et al. (1995) Eur. J. Immunol. 25:2433-2437), NKG2C and NKG2E (Houchins, et al. (1991) J. Exp. Med. 173:1017-1020), and ILT1 (Samaridis and Colonna (1997) Eur. J. Immunol. 27:660-665) receptors is the presence of a basic amino acid (K or R) in the transmembrane domain. Given the precedent for interactions between proteins of multi-subunit receptor complexes via oppositely charged amino acids in the transmembrane domains, e.g. the CD3/TcR complex (Chan, et

al. (1994) Ann. Rev. Immunol. 12:555-592), we examined whether DAP12 associates with the non-inhibitory KIR2DS2 glycoprotein containing a K in the transmembrane region (Colonna and Samaridis (1995) Science 268:405-408). The murine Ba/F3 pre-B cell line was transfected with a cDNA encoding KIR2DS2 either alone or together with a DAP12 cDNA containing a FLAG epitope tag at the N terminus to permit detection with an anti-FLAG mAb. Transfectants were selected by flow cytometry for cell surface expression based on positive staining with anti-KIR mAb DX27 or anti-FLAG mAb M2. KIR2DS2 Ba/F3 and KIR2DS2 + DAP12-FLAG Ba/F3 transfectants were surface labeled with ^{125}I, lysed in 1% digitonin to preserve non-covalent associations of membrane protein complexes, and immunoprecipitated with anti-KIR mAb or anti-FLAG mAb. The tyrosine residue in the FLAG epitope provided a site for radioiodination, permitting visualization of the DAP12 protein. Anti-KIR mAb immunoprecipitated an ^{125}I labeled species of ~50-60 kD from both the KIR2DS2 Ba/F3 cells and KIR2DS2 + DAP12-FLAG Ba/F3 transfectants, consistent with the predicted molecular weight of the KIR2DS2 glycoprotein. An additional ^{125}I labeled protein of ~12 kD was co-immunoprecipitated with anti-KIR mAb from the KIR2DS2 + DAP12-FLAG transfectant, but not from the transfectant expressing only KIR2DS2. Reciprocally, an ^{125}I labeled glycoprotein migrating identical to KIR2DS2 was co-immunoprecipitated with anti-FLAG mAb from the KIR2DS2 + DAP12-FLAG Ba/F3 cells, but not from the KIR2DS2 only transfectant. Comparison of immunoprecipitates analyzed by SDS-PAGE using either reducing or non-reducing conditions indicate that DAP12 is expressed on the cell surface as a disulfide-bonded dimer. It should be noted that we were unable to detect cell surface expression of DAP12 on the surface of Ba/F3 cells transfected with the DAP12-FLAG cDNA alone, without KIR2DS2. However, DAP12-FLAG proteins were detected in

the cytoplasm, suggesting that DAP12 may require association with its partner subunits for efficient transport to the cell surface, similar to the situation with the CD3 proteins (Clevers, et al. (1988) Ann. Rev. Immunol. 6:629-662). Additionally, preliminary results indicated that DAP12 does not associate with the inhibitory KIR isoforms that lack a charged residue in their transmembrane domain.

A peptide corresponding to the cytoplasmic domain of DAP12 (ITETESPY*QELQGQRSDVY*SDLNTQRP; see SEQ ID NO: 2) was synthesized either as an unphosphorylated protein or containing phosphates on both Y residues. Lysates from Jurkat T cells or NK cell clone A6 were incubated with the biotinylated peptides and complexes precipitated using avidin-agarose. Western blot analysis demonstrated that a DAP12 peptide phosphorylated on both Y residues, but not the unphosphorylated peptide, formed complexes with the ZAP-70 kinase. The tyrosine phosphorylated DAP12 peptide, but not the unphosphorylated DAP12 peptide, also formed a complex with the Syk protein tyrosine kinase in lysates from NK cells. The binding of these kinases to phosphorylated DAP12 is remarkably reminiscent of the interactions that have been demonstrated between the phosphorylated ITAM-containing CD3 subunits and Syk or ZAP-70 kinases during TcR signaling. Iwashima, et al. (1994) Science 263:1136-1139; and Chan, et al. (1994) J. Immunol. 152:4758-4766.

Ligation of the CD3/TcR complex on T cells or the Ig receptor complex on B cells resulted in cellular activation. Therefore, studies were undertaken to examine the functional consequence of cross-linking the KIR2DS2-DAP12 complex. Ba/F3 transfectants expressing either KIR2DS2 alone or the KIR2DS2-DAP12-FLAG complex were incubated with anti-KIR mAb DX27 or anti-FLAG mAb, followed by a goat anti-mouse Ig to provide cross-linking. Examination of total cellular proteins in Ba/F3

cells expressing the KIR2DS2-DAP12-FLAG complex that were stimulated with anti-KIR or anti-FLAG mAb revealed tyrosine phosphorylation of several cellular substrates. Immunoprecipitation with anti-FLAG mAb and Western blot analysis with anti-phosphotyrosine mAb demonstrated that cross-linking the KIR2DS2-DAP12-FLAG transfectants with anti-KIR mAb induced tyrosine phosphorylation of the DAP12 protein and resulted in the association of phosphorylated DAP12 with the Syk protein tyrosine kinase. By contrast, Ba/F3 cells expressing only KIR2DS2 were not activated by cross-linking with anti-KIR mAb. Similarly, up-regulation of CD69 expression was observed in Jurkat T leukemia cells transfected with both KIR2DS2 and DAP12, but not KIR2DS2 alone, when these receptors were cross-linked with anti-KIR mAb. These results indicate that DAP12 is necessary and responsible for KIR2DS2 signal transduction in these host cells and are in accordance with prior observations demonstrating that KIR2DS2 molecules are functional in NK cells, but not in transfectants expressing only KIR2DS2. Bléry, et al. (1997) J. Biol. Chem. 272:8989-8996.

These studies suggest that DAP12 may associate with the non-inhibitory isoforms of the KIR molecules in NK cells and permit cellular activation via these receptors, similar to the function of the CD3 subunits in the TcR complex and CD79 subunits in the B cell receptor complex. Expression of DAP12 in monocytes and dendritic cells predicts association with other receptors similar to the non-inhibitory KIR present in these cell types. Likely candidates are the recently identified ILT/MIR family of molecules expressed by human monocytes (Wagtmann, et al. (1997) Current Biology 7:615-618; and Samaridis and Colonna (1997) Eur. J. Immunol. 27:660-665) and the PIR-A molecules in rodent myeloid and B cells (Hayami, et al. (1997) J. Biol. Chem. 272:7320-7327; and Kubagawa, et al. (1997) Proc. Natl. Acad. Sci. USA 94:5261-5266). In

XVIII. Isolation of Associated Proteins

DAP12 remains localized intracellularly when expressed in cells in the absence of associating partners. This observation was exploited with the purpose of cloning novel DAP12-associating proteins, e.g., to expression clone genes necessary in the process of cellular localization to the membrane. Cells lacking the associated proteins were transfected with the DAP12, and the protein remained intracellularly localized. These cells could be used to expression clone necessary accessory proteins for DAP12 surface localization. The strategy had been labeled "DAP-trap".

To this end, a FLAG-tagged form of mouse DAP12 was expressed in 293T cells using an expression vector, e.g., pREP10. In the presence of hygromycin, a stable DAP12 expressing cell line was selected, DT381. To reduce the background of spontaneous DAP12 expression at the cell surface, DT381 cells were negatively selected by flow cytometry using the M2 anti-FLAG mAb (Kodak). To clone novel DAP12 associating proteins, a J774 macrophage cell line derived pJEF14 expression library was transfected into DT381 cells. Forty-eight hours after transfection, the cells were selected for cell surface expression of DAP12 by flow cytometry. This was performed by two color staining: DAP12 was visualized using the M2 anti-FLAG mAb, followed by a biotin-conjugated anti-mouse IgG1 mAb (#02232D Pharmingen), followed by a streptavidin-PE third step incubation. Fc receptors on transfected DT381 cells were visualized using the directly FITC-conjugated anti-CD16/32 mAb 2.4G2 (#01244D Pharmingen). Only single PE positive cells were sorted. Staining with the anti-CD16/32 mAb was necessary to avoid the cloning of Fc receptors which are abundantly present in J774 cells.

The plasmids from the sorted cells were rescued and the DNA was retransformed into DH10B bacteria.

Sublibraries were obtained and subjected to a novel round of expression cloning. After three rounds of selection, 500 single bacterial colonies from the third sublibrary were grown in a 96 well plate format to construct a three dimensional matrix of consisting of 5 x 12 x 8 colonies. DNA obtained from pools of each X, Y, and Z coordinate of this matrix was again transfected into DT381 cells and the transfectants were screened for DAP12 surface expression.

This resulted in the identification of two identical clones, both encoding a 165 amino acid type II transmembrane protein of the C-type lectin superfamily. This gene/protein was designated Myloid DAP12 associating Lectin-1 (MDL-1). This embodiment of MDL-1 from the mouse has an intracellular region of 2 residues, a transmembrane region of 23 residues, and a 140 residue extracellular region containing the C-type lectin domain. The transmembrane segment possesses a charged amino acid, an the extracellular region has three putative N-glycosylation sites. BLAST searching revealed a highly homologous full length mouse EST, AA186015, which was identical to the two above mentioned clones, with the exception that this clone has an extra stretch of 75 nucleotides resulting in a 25 residue additional stretch extracellularly just outside of the transmembrane region. Thus, there exist two embodiments, a short form and long form. The rest of the sequences are identical.

Searching within a DNAX sequence database revealed a homologous human EST, #97-1128A12, which encodes a human homologue of MDL-1. The mouse MDL-1 appears to be encoded by a single gene, in contrast to many related surface proteins, which may occur in families of genes. The mouse MDL-1 expression is restricted to monocytes, macrophages and dendritic cells.

Because the MDL-1 gene appears to be crucial in localization of the DAP12 to the membrane, and possesses

interesting structural features, it is likely that the MDL-1 associates with the DAP12 in a membrane complex. Thus, disruption of the complex may lead ton interesting blocking of function of the DAP12-receptor complex. This suggests obvious approaches to small molecule drug screening for compounds which would interfere with association. Alternatively, transmembrane fragments may block functional association. Antibodies to the extracellular regions, either of proteins alone, or the combination of components in the functional complexes, would be useful in diagnostic or therapeutic contexts.

DAP10 also seems to associate with an accessory protein. In particular, immunoprecipitation of DAP10 under mildly denaturing conditions results in co-immunoprecipitation of a protein band of about 40-41 kD. Neuraminidase treatment, or O-glycanase treatment, result in a decrease in molecular weight to about 38-39 kD. N-glycanase treatment causes a decrease in molecular weight to about 28-30 kD. These suggest that the protein is about 26-30 kD without glycosylation. Standard or microsequencing methods can be applied to protein isolated by immunoprecipitation. With sequence, redundant PCR primers, or other techniques can be applied to isolate the gene. Alternatively, sequence may allow identification of the gene by matches in sequence databases.

Moreover, the DAP10 is also subject to the DAP-trap strategy. Expression cloning techniques can be applied, as with the DAP12, to clone the gene from a cDNA library. Distribution information will allow selection of the appropriate cell lines and cDNA libraries for such.

All citations herein are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.